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Effects of the carcinogenic polycyclic aromatic hydrocarbons (PAH), benzo[a]-pyrene (BAP), and methylcholanthrene (MCA) were investigated with sheepshead minnows (Cyprinodon variegatus) and channel catfish (Ictalurus punctatus). A closed-circulating system was designed to maintain up to 100 sheepshead minnows in artificial seawater for longterm exposures. Fish were maintained in this system for up to 31 weeks with weekly contaminations of PAH. Due to their chemical properties significant levels of BaP and MCA remained in the water column for only ca. 24 hours each week and no tumors were observed in the exposed fish during the period of the study.

The incidence and types of lesions in control and exposed fish were basically similar except in catfish that were fed PAH contaminated food. High levels of contamination (1mg/gm food) appeared to be toxic and lower levels of contamination (0.1 mg/gm food) produced sufficient stress to make the catfish susceptible to fatal parasite infestations. Both species accumulated radioactively labelled PAH at concentrations much higher than their nominal concentraions in the water.

These results demonstrate that sheepshead minnows function well as experimental organisms in artificial seawater in a closed system maintained at a noncoastal facility. Thus, they provide an excellent model system for the study of longterm effects of chronic exposure to polluting agents.

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EFFECTS OF PETROLEUM COMPOUNDS ON ESTUARINE FISHES

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FOREWORD

The protection of our estuarine and coastal areas from damage caused by toxic organic pollutants requires that regulations restricting the introduction of these compounds into the environment be formulated on a sound scientific basis. Accurate information describing dose-response relationships for organisms and ecosystems under varying conditions is required. The EPA Environmental Research Laboratory, Gulf Breeze, contributes to this information through research programs aimed at determining:

- the effects of toxic organic pollutants on individual species and communities of organisms;
- the effects of toxic organics on ecosystem processes and components;
- the significance of chemical carcinogens in the estuarine and marine environments.

Considerable interest has focused recently on the fate and possible effects of carcinogens and mutagens in the aquatic environment which usually is the ultimate receptacle for pollutants. This report presents the design and results of use of a closed system carcinogen assay apparatus long-needed by investigators remote from flowing-water laboratory facilities. Further, the fate and some possibly long-term effects of polycyclic aromatic hydrocarbons in the marine estuarine environment and biota are described. This data may serve to alert us to the role of certain carcinogens in the environment.

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ABSTRACT

The overall goal of this project was to study the effects of the carcinogenic polycyclic aromatic hydrocarbons (PAH), benzo(a)pyrene (BaP) and methylcholanthrene (MCA), on sheepshead minnows (Cyprinodon variegatus) and channel catfish (Ictalurus punctatus).

A closed-circulating system was designed to maintain up to 100 sheepshead minnows in artificial seawater for long-term exposures. Fish were maintained in this system for up to 31 weeks with weekly contaminations of PAH. Due to their chemical properties, significant levels of BaP and MCA remained in the water column for only ca. 24 hours each week, and no tumors were observed in the exposed fish during the period of the study.

The incidence and types of lesions in control and exposed fish were basically similar except in catfish that were fed PAH contaminated food. High levels of contamination (1 mg/gm food) appeared to be toxic, and lower levels of contamination (0.1 mg/gm food) produced sufficient stress to make the catfish susceptible to fatal parasite infestations.

Both species accumulated radioactively labelled PAH at concentrations much higher than their nominal concentrations in the water. Although the level of accumulation was extremely variable, in general the accumulation factors were: ca. 30X in gill and liver, ca. 15X in GI tract, and ca. 2X in skeletal muscle.

In summary, these results demonstrate that sheepshead minnows function well as experimental organisms in artificial seawater in a closed system maintained at a noncoastal facility. Thus, they provide an excellent model system for the study of long-term effects of chronic exposure to polluting agents. The sheepshead minnow, widespread in Gulf estuaries, therefore provides an excellent indicator organism that can be used to make extrapolations from the laboratory to the feral population.

Future studies should concentrate on tumor induction tests with known or suspected carcinogens in the system established in this study.

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ABBREVIATIONS AND SYMBOLS

BaP	-- benzo(a)pyrene
BaP-H	-- 1 milligram benzo(a)pyrene per gram food
BaP-L	-- 0.1 milligram benzo(a)pyrene per gram food
gm	-- gram or grams
l	-- liter or liters
mg	-- milligram or milligrams
MCA	-- 20-methylcholanthrene
MCA-H	-- 1 milligram methylcholanthrene per gram food
MCA-L	-- 0.1 milligram methylcholanthrene per gram food
ng	-- nanogram or nanograms
PAH	-- polycyclic aromatic hydrocarbons
µg	-- microgram or micrograms
ppb	-- parts per billion

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SECTION 1

INTRODUCTION

Reliable estimates that 75% to 90% of the incidence of human cancer may be environmentally related increased public awareness of the importance of environmental pollutants as tumorigenic agents (1,2). This has resulted in demand for more rigorous environmental safety assessment (3,4). Our current methods for detecting chemical carcinogens and capability of accurately predicting the human health hazard resulting from a particular chemical agent do not provide the degree of safety assessment needed (3,5). The Ames quick detection technique (6) is an example of an important advance in methods of assessment; however, long-term bioassays continue to be our most reliable method of testing a chemical for carcinogenicity (4).

Murine assay systems, our mainstay for testing carcinogens, are not particularly amenable to assessment of the aquatic environment. Since the aquatic environment becomes a "sink" for many potentially dangerous pollutants, it seems imperative that valid test systems be developed.

Teleost fishes, ubiquitous in the aquatic environment, are obvious candidates for a monitoring role. Changes in incidence of tumors in feral fish populations could provide a built-in "early warning" of the presence of a carcinogen. Evidence already exists that such correlations between level of pollution and incidence of proliferative diseases may be possible (7-10). Fish make excellent experimental animals for these tests because they are immersed in an aquatic environment that can be easily manipulated experimentally. Also, they grow throughout a relatively short life span, and yet many species remain small in size. This small size and the apparently short latency period for tumor induction (11) are important factors in reducing test costs.

As the levels of pollution have increased dramatically in many aquatic ecosystems throughout the world, there has been an apparent increase in the detection of proliferative diseases in aquatic organisms (12). Carcinogenic hydrocarbons are found at high levels in some aquatic systems; however, currently their levels remain relatively low in many of the estuaries of the Gulf Region. This condition is likely to change in the near future if predicted rapid increases in population and industrialization occur. The quality of this environment is likely to be further threatened due to existing plans to dramatically increase petroleum imports through these waters.

The major goal of this project was to conduct a comprehensive study of the effects of two carcinogenic hydrocarbons, benzo(a)pyrene (BaP) and methylcholanthrene (MCA), on the sheepshead minnow (Cyprinodon variegatus) and the

channel catfish (Ictalurus punctatus), two teleost species indigenous to the Gulf Region. The project provides important background data supporting the feasibility of using teleost fishes in the assessment of suspected carcinogens. Furthermore, it represents a contribution to the development of new methodologies critically needed (3) to facilitate the reliable, rapid, and inexpensive assessment of the ever-growing avalanche of new and possibly carcinogenic compounds that enter the aquatic environment (13).

SECTION 2

CONCLUSIONS AND RECOMMENDATIONS

The closed-circulating system designed for the maintenance of sheepshead minnows in artificial seawater has proven to be adequate for keeping as many as 100 fish in good health indefinitely. The system can be easily maintained and contaminated with minimum risk to laboratory workers. Seasonal mortality problems experienced during the adaptation period could likely be solved by raising fish from eggs under laboratory conditions. Large numbers of sheepshead minnows can be produced in this manner (14), and the possibility of bringing in parasites from the feral environment would also be eliminated. In fact, the increasing use of the sheepshead minnow as a laboratory animal (15-17) suggests that it might be advisable to develop a laboratory strain as is the case for rats and mice.

The chemical properties of BaP and MCA made it difficult to maintain a high level of exposure or to accurately evaluate the concentration of PAH within the water column. Less than 25% of the concentration of BaP placed in the system remained in the water column after 24 hr, and less than 5% can be found shortly thereafter. Although MCA can be found at higher levels initially, it essentially disappears from the water column in an equally short time. Consequently, weekly contamination provided only one day in seven of significant exposure.

These results suggest that feral fish are unlikely to be exposed to high levels of PAH in the water column except in the immediate area of a constant effluent high in PAH content. It appears that fish are more likely to be contaminated with PAH as a result of their feeding habits. The fact that fish accumulated PAH and/or their metabolities provides a mechanism by which PAH could get into the aquatic food chain and suggests that fish from areas of high PAH contamination should be monitored for PAH content prior to human consumption.

The task of establishing a cause-effect relationship between PAH and the types and incidence of diseases in these fish is a difficult one, and considerable additional work must be accomplished before valid conclusions can be reached. The project provides evidence that the ingestion of PAH can produce enough stress to render fish susceptible to parasites and disease. Thus, in this indirect way, PAH could have serious effects on production and the biological success of a species. The data provide some evidence that PAH exposure may contribute to the conditions of lordosis and/or scoliosis and possibly nervous disorders in channel catfish. A detailed study of electrolyte physiology and the morphology of the tissues important in electrolyte control (i.e., Ultimobranchial gland and Stannius gland) of both exposed and control fish could possibly provide some clarification concerning this matter.

The apparent wide variability in the accumulation of label by fish exposed to radioactively labelled PAH needs further study. It would be useful to study fish that have been fed labelled PAH. Biochemical studies to disclose the amount of parent compound and the amounts and chemical nature of the PAH metabolites accumulated will be necessary to determine the real significance of these studies. A study of the photochemical reactions occurring in the system would also aid our understanding of the results.

Development of fish tissue culture cell lines as carcinogen assay systems may seem somewhat aside from the main thrust of the project; however, they provide an additional system for the study of neoplastic mechanisms at the cellular level. The tissue culture exposures are likely to be particularly applicable to screening for mutagenic effects. The development of nonfibroblastic cell lines from sheepshead minnow embryos might also provide a valuable tool for this same purpose. Since an organism should be most vulnerable to carcinogenic and mutagenic agents at times of rapid cell division, experiments exposing sheepshead minnows to such agents during embryonic development should be conducted.

Finally, the results of the project demonstrate that the sheepshead minnow can be maintained in good health in a closed system for the extended periods necessary to study the long-term effects of chronic exposure to a polluting agent. Thus, the sheepshead minnow in this system provides a suitable model for chronic testing and should be extensively employed for this purpose. Since the sheepshead minnow is common in the estuaries of the Gulf and Atlantic, data obtained concerning this fish in the laboratory should be useful in making reliable extrapolations concerning conditions in the estuaries by observing samples from the feral population. Therefore, it is recommended that efforts be continued to develop extensive baseline data concerning the incidence of neoplastic and other lesions at selected sites throughout the Gulf Region. These baseline data plus data from laboratory exposures under controlled conditions should provide a valuable "early warning mechanism," making possible the discovery and removal of a chemical insult before it produces serious and long-term effects.

SECTION 3

MATERIALS AND METHODS

Wet Laboratory

In order to provide safe and adequate facilities for working with carcinogens, the University of Southern Mississippi expended approximately $17,000 to modify an existing brick facility located four miles from the main campus. This construction provided a quality wet laboratory that is functional and safe enough for long-term involvement in aquatic research. The laboratory allows control of the ambient environment and provides for the safe handling and disposal of contaminated water.

Specimen Collection

During the course of the study, approximately 6500 sheepshead minnows (Cyprinodon variegatus) were collected. Approximately 5000 were seined from a marshy tidal entrance at Range Point on Santa Rosa Island near the U.S. EPA Environmental Research Laboratory, Sabine Island, Gulf Breeze, Florida. Approximately 1500 sheepshead were collected from a similar tidal stream on the north side and at the east end of Horn Island on the Mississippi Gulf Coast near Pascagoula, Mississippi. Fish were collected during each month of the year except January and February. All specimens were examined for gross lesions and treated for 30 minutes with 1:4000 formalin to remove parasites. The fish were acclimated to 5 to 15 $^{o}/_{oo}$ artificial seawater (Rila Mix, Rila Products, Teaneck, NJ) at least 5 days prior to being placed in an experimental system.

Approximately 1400 Channel catfish (Ictalurus punctatus) were obtained as 1- to 3-inch fingerlings from local commercial fish hatcheries during the course of the study. These fish were also formalin-treated prior to their use in experimental systems.

The sheepshead minnows were fed a diet prepared by mixing 400 grams of cat food (Kozy Kitten brand) with 250 gm of No. 3 Purina Trout Chow. The mixture was pelletized by running it through a meat grinder, prepared weekly, and kept under refrigeration until used. The channel catfish were fed either the catfood-trout chow mixture or commercial catfish food (Purina). Both species were fed approximately 3% of their body weight per day.

Exposure Systems

Sheepshead minnows were maintained in closed-circulating systems

consisting of 5 aquaria constructed of double-thick glass or fiberglass-coated 1/2-inch exterior plywood with a double-thick glass front (Fig. 1). The aquaria have PVC stand pipes that provide a standing water capacity of 150 liters. The aquaria drain into a common subtank. The subtank is constructed so that water is forced up through a filter system into a water holding compartment (Fig. 2). The holding compartment contains a submersible pump (Little Giant, 3E-12NDVR) controlled by a float valve system (IP504 Automatic Float Switch, W. H. Grainger, Inc., New Orleans, LA) that intermittently pumps the water into a head box placed above the five aquaria. Five drains in the bottom of this box allow water to gravity flow into the aquaria. The drains are fitted with rubber stoppers that contain holes. Thus, the flow rate of the system is a function of total water volume and the size of the holes in the rubber stoppers.

Most of the experiments with catfish were conducted in 950-liter cylindrical fiberglass tanks (Reeves Plastic Engineering, Pascagoula, MS). These tanks contain Venturi lifters and can be operated with constant or intermittent flow-through to maintain water quality. Some experiments involving both catfish and sheepshead minnows were conducted in 185-liter capacity rectangular fiberglass-coated plywood tanks equipped with bottom drains and stand pipes. Both types of tanks were provided compressed air through air stones. External filters were employed with 185-liter tanks.

PAH Contamination of Water

Acetone was used as a carrier for the introduction of PAH into the systems. To obtain the 10 ppb exposures, 6 ml of acetone containing 1.5 mg PAH per ml was added to the systems. The 50-ppb exposures were obtained by adding 10 ml of acetone containing 4.35 mg PAH per ml to the systems. Equal volumes of acetone were added to controls. The contaminant was always added to the holding compartment of the subtank (Fig. 2).

Contaminants were added to the tanks in a similar manner for the catfish exposure experiments.

Chemical Analysis

The concentrations of BaP and MCA in the exposure system water were determined by two different methods. Initially, the PAH were extracted from one-liter water samples with 100 ml of hexane. Interference due to high boiling aliphatic hydrocarbons was eliminated by evaporating the hexane to a 1 ml volume, adding it to a column of 1 g neutral alumina over 1 g silica gel and eluting the aliphatic hydrocarbons with 10 ml hexane. The BaP (benzo(a)-pyrene) and MCA (methylcholanthrene) was then eluted with 10 ml of methylene chloride/hexane (50/50, V/V), evaporated to 1 ml or less, and analyzed by gas chromatography with flame ionization detectors (GC-FID). A 3mm x 2m stainless steel column packed with 3% SP-2100 on Supelcoport 80/100 was utilized with a temperature programmed from 150 to 250°C at 4°/mm. Two inherent difficulties were experienced with this procedure. In some cases, the removal of the

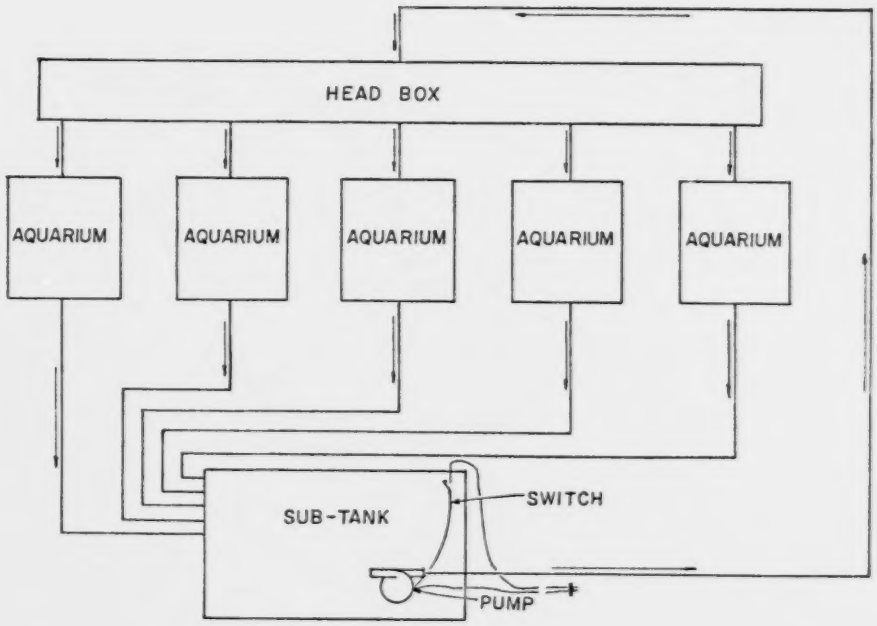

Figure 1. Schematic diagram of the basic components and the waterflow in the closed-circulating system. The arrows indicate the direction of water flow. The switch is controlled by a float mechanism as the water level changes in the sub-tank. The head box, aquaria, and the sub-tank are all completely covered to minimize air contamination.

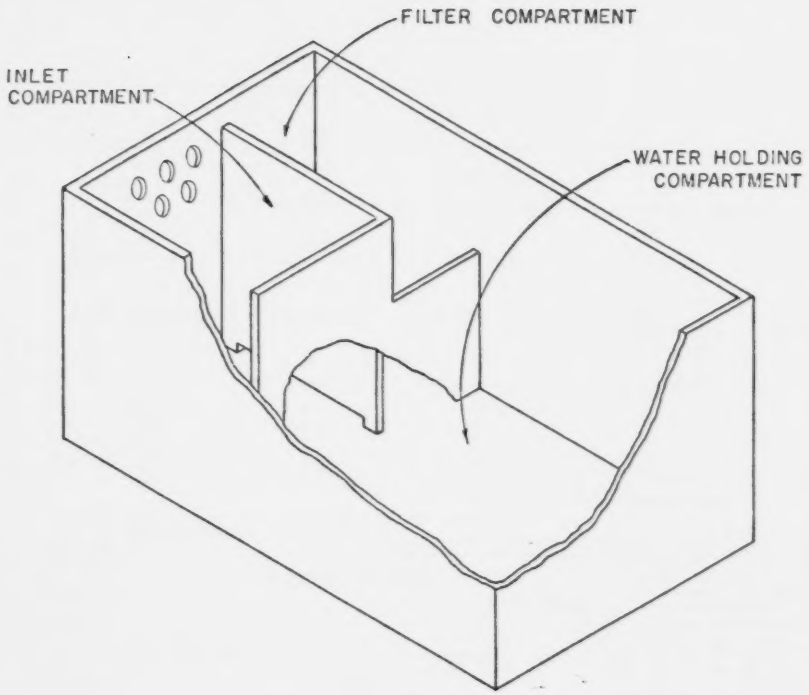

Figure 2. Detailed structure of closed-circulating system sub-tank. The five holes in the wall of the inlet compartment are the points of attachment for the hoses from the aquaria. The filter compartment contains a false bottom not illustrated that supports the filter material 3 inches above the bottom of the sub-tank. This allows water to flow from the inlet compartment, up through the filter material and into the holding compartment. The submersible pump is placed in the water holding compartment and the float controlled switch is mounted on the wall of this compartment.

required number of one-liter samples represented an appreciable amount of the total volume of water in the system, and more importantly, the procedure, did not provide the sensitivity needed for the low concentrations of contamination encountered.

A second more sensitive procedure that employs HP liquid chromatography was used to analyze most of our samples (18). Due to the increased sensitivity of this procedure, 10 to 100 ml water samples were sufficient for analytical purposes.

PAH Feeding Experiments

For the high concentration of contaminate (1 mg/gm feed), BaP (BaP-H) or MCA (MCA-H) was dissolved in 20 ml of acetone, blended with 257 gm of feed, and then pelletized. The low concentration of contaminate (BaP-L or MCA-L) was prepared in a similar manner but with only 0.1 mg PAH/gm feed.

The contaminated food was fed once each day at the rate of approximately 3% of body weight per day.

Bioaccumulation Studies

Fish were placed in 38 liter aquaria and allowed to adapt for two days. Then 0.08 µg/l of ^{3}H-benzo(a)pyrene or 0.037 µg/l of ^{3}H-20 methylcholanthrene (Amersham Corp.) were added to the system. Gill, liver, G.I. tract, and skeletal muscle tissues were excised, weighed, and homogenized in chloroform: methanol (3:1). The filtrate from a glass wool-filled pipette was then evaporated to dryness: scintillation cocktail (1 liter toluene:4 gPPO) was added to the vial and the filtrate was counted in a Packard Tri-Carb Liquid Scintillation Spectrometer.

Tissue Culture

A sheepshead cell line (SHF) developed from cultured fin fibroblasts of a male Archosargus probatocephalus was employed for these studies (19). C-band staining was accomplished by modifying the procedure described by Howard et al. (20) for human leukocyte cultures. The incubation time was increased from 24 to 48 hours and the staining time for the slides in Giemsa stain was increased to 15 minutes. This modified staining procedure was also applied successfully to preparations of cell lines from spleen fibroblasts of the silver perch, Bairdiella chrysura (21), and fin fibroblasts of the salt-water blue striped grunt, Haemulon sciurns (22).

Chromosomes of the SHF cells were prepared for karyotyping by a modification of the method of Sumner et al. (23). Colcemid was added to the cultures to a final concentration of 0.25 µg/ml for 2-3 hours. The medium was then removed and the flasks rinsed with trypsin-versene$^{(R)}$ (ATV) (24). The ATV treatment was repeated until all the cells were removed. The pooled cell suspension (original medium and all rinses) was centrifuged at 1000 rpm

for 10 minutes, the supernatant was aspirated off, and the cellular pellet was resuspended and treated for 20 minutes at room temperature with a hypotonic solution of 0.075 M KCl. Fixative (3 parts absolute methanol: 1 part glacial acetic acid) was added and the cells were pelleted again by centrifugation. The fixed cells were dropped on clean dry slides and allowed to air dry prior to staining in a standard Giemsa stain.

Histological Preparations

Tissues prepared for light microscopy were fixed in Davidson's fixative (25) in the cold for 24 to 96 hours. Fixed tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin according to routine procedures.

SECTION 4

RESULTS AND DISCUSSION

Closed-circulating Exposure Systems

Increasing utilization of marine organisms such as the sheepshead minnow in bioassays and laboratory studies of the effects of pollutants (14-17) creates a need for closed systems in which reasonably large numbers of animals can be maintained in good health. In addition to the need to study marine organisms in inland laboratories, closed systems are needed at marine laboratories to avoid releasing large amounts of dangerous and sometimes costly chemicals into the estuarine environment.

The closed-circulating systems designed for this project provide a relatively inexpensive solution to this problem. They are constructed of low-cost, easily available materials and designed to be easy and safe to operate. The system is essentially enclosed yet needs no external aeration, thus reducing the risk of aerosol contamination. The design of the subtank provides both mechanical and biological filtration by a filter system that can be charged with oyster shell, charcoal, or any other commonly used filtration material (Fig. 2). Overflow water from the aquaria flows up through the filter from the bottom of the subtank into the water holding compartment, causing debris to accumulate in the bottom of the subtank. Consequently, at the time periodic water changes are made by draining the subtank, this debris can be easily washed out through the drains. Also, water can be run "backwards" through the filter material for additional cleaning with little risk of operator contamination. In our experience 75 to 125 adult sheepshead minnows can be maintained indefinitely with good water quality by draining the subtank once each week. When the system is stocked and functioning properly, nitrites have averaged 0.123 ppm, nitrates 18.8 ppm. Although we have experienced problems obtaining reliable readings with our ammonia test, ammonia seems to stay reasonably low. The pH averages 8.5 and rarely drops below pH 8.3. These values are within the quantitative ranges considered ideal for marine aquaria (26).

Our experience indicates that it is extremely important that the fish be healthy, stress free, and relatively clear of parasites when they are placed in the system. If these conditions are met, an acceptable survival rate can be expected over an extended period (Table 1).

The importance of the state of health of the feral fish may be illustrated by the fact that during each year of the study sheepshead minnows collected in the early spring died in large numbers before they could be adapted to laboratory conditions. Experiment 5 in Table 1, which is the

lowest rate of survival experienced, were survivors of a group of fish caught at this time of year.

A caution concerning the operation of the system is that care must be taken to see that an adequate water level is maintained at all times since circulation will stop when the water falls below a critical level. Additionally, the brass rod on the float valve switching device must be cleaned and freed of corrosion periodically if the switch is to function properly. (An effort is currently underway to redesign the switching system to alleviate this problem.)

TABLE 1. LONG-TERM EXPOSURES OF SHEEPSHEAD MINNOWS

Experiment Number	Number of Fish	Contaminant[1]	Length of Exposure (wks)	Percent Survival
1	100	Control	10	70
2	125	1 µg/l PaP	25	66
3	125	10 µg/l MCA	13	100
4[2]	125	50 µg/l MCA	31	86
5	45	50 µg/l MCA	15	44

[1]Contaminated once each week.
[2]Continuation of experiment No. 3 with an increase in level of contamination.

Chemical Analysis

Before relationships can be established between tumor incidence and the concentration and mode of exposure to a tumorigenic agent, it is necessary to establish a profile of the actual concentrations of the agent in the water column throughout the time course of the experiment. Table 2 provides data concerning the levels of BaP at different points in the closed-circulating system for a six-day period after the system was contaminated with 10 µg/l. Since much of the BaP precipitates and floats on the water surface, the values determined shortly after contamination will vary greatly according to the method of sampling. Consequently, samples were taken with a pipette ca. 27 cm below the water surface. The data in Table 2 indicate that BaP is in the water column of the subtank at a level of about 80% of the theoretical level of contamination. The concentration in the different aquaria is somewhat varied at one hour; however, the mean value for the aquaria at this time is about 35% of the theoretical concentration (Fig. 3). Although we had expected large amounts of PAH to adhere to the filter surfaces, the concentration of BaP in the effluent from the filter one hour after exposure is actually higher than the mean for the five aquaria, suggesting that the filter

did not significantly lower the concentration. After 24 hours the level of BaP at all sampling points had dropped rapidly to about 20% of the theoretical concentration. From the 4th to the 6th day, the concentration dropped to about 5% (Fig. 3). These data indicate that weekly contamination of the system with BaP at a level of 10 µg/l provides a significant amount of BaP in the water column for only the first one or two days of the exposure period.

TABLE 2. BaP CONCENTRATION IN CLOSED-CIRCULATING SYSTEM[1]

	Time of Sampling			
	1 hr	24 hr	96 hr	144 hr
Sampling Location				
Holding Compartment of Subtank	8.0[2]	2.2	0.4	0.3
Filter Effluent	4.5	2.1	0.5	0.5
Aquarium - 1	2.6	1.6	0.4	0.3
Aquarium - 2	3.0	1.6	0.5	0.3
Aquarium - 3	5.7	2.6	0.5	0.5
Aquarium - 4	3.1	2.0	0.3	0.3
Aquarium - 5	3.2	1.8	0.5	0.4

[1]System contaminated at the beginning of the experiment with 10 µg BaP per liter of water.
[2]All values in µg/l (ppb).

Somewhat similar results were obtained when the system was contaminated on a weekly basis with BaP at a concentration of 50 µg/l for a three week period (Table 3). These data provided no evidence for week-to-week accumulation of BaP in the system over the three-week period since the concentrations for the second and third weeks were not significantly higher than those observed for the first week. Also, the profile of concentration during each week was similar to that observed at the 10 µg/l contamination. For example, one hour after contamination the mean concentration was about 45% and by 24 hours it had dropped to 22%. By the 4th day, the concentration had leveled off at 2-3%.

When the systems were contaminated each week with 50 µg MCA/l for five weeks, a similar profile of concentrations resulted; however, the absolute

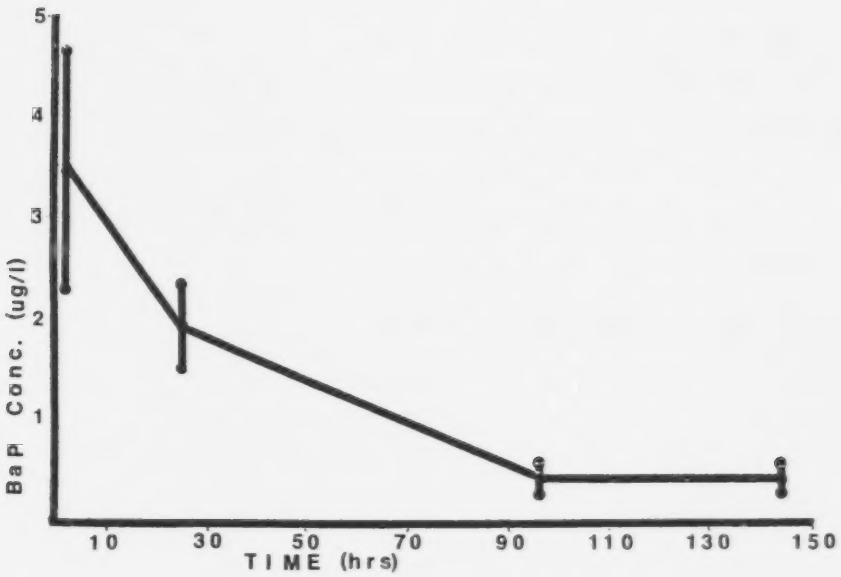

Figure 3. Profile of BaP concentration in closed-circulating system over a six-day period. Initially, the system was contaminated with 10 µg of BaP per liter of water. The graph indicates means ± SE of the values from aquaria 1 through 5 of the system.

values were higher (Table 4). As in the case of BaP contamination the evidence does not indicate an accumulation of MCA in the system over the five-week period. The concentration one hour after contamination was 65.3 µg/l, a value which is higher than the 50 µg/l theoretical level of contamination. This may seem surprising; however, it is a common occurrence immediately after contamination before the contaminant has become dispersed throughout the whole system. At 24 hours after contamination, the concentration is 47.7 µg/l which is 95% of the theoretical concentration, and by the 4th and 6th days, the values had dropped to 12% and 10%, respectively.

These data suggest that MCA is more soluble in aqueous solutions than BaP. Thus, when 50 µg/l contamination of BaP and MCA are compared one hour after contamination, the concentration of BaP in the water column is less than 25% of that observed for MCA, and this relative difference remains essentially the same even after 4 to 6 days. Perhaps this higher solubility which makes MCA more accessible to organisms and cells in an aqueous environment is one reason why MCA is sometimes listed as a more potent carcinogen than BaP (27).

Because of the low solubility properties of both BaP and MCA, it seems that a significant portion of these PAH would remain in the water column for only a transient period. Thus, weekly contaminations produce "spike" type exposures typical of what might occur when PAH-containing wastes are repeatedly released into the aquatic environment in discrete amounts (28). This method of laboratory exposure is therefore a good model for the type of pollution that occurs in ocean dumping of petroleum and in some types of industrial effluents.

The relative insolubility of these PAH suggests that they and similar PAH may not cause serious and widespread problems for fish due to direct contact with the compound in the water column. However, since PAH may accumulate in somewhat higher concentrations in some marine invertebrates and bottom sediments (27), it is feasible that some fish, as a result of their feeding habits, may be exposed to high levels of PAH through ingestion. Even if in the final analysis PAH does not prove to be a serious carcinogenic agent in marine fish and invertebrates, it is of obvious significance that when exposed to PAH, fish and invertebrates such as the American oyster (Crassostrea virginica) (28), an important human food item, is known to accumulate these potent mammalian carcinogens.

Long-term Exposures

During the course of the study, we have maintained a full complement of Cyprinodon in our systems for a total of 94 weeks with the same group of fish in the system for as long as 25 weeks (Table 1). During this time a 73% overall survival rate occurred. No obvious lesions were observed in about 20% of the sheepshead minnows sacrificed and examined because they showed signs of illness or were moribund. However, of the remainder that presented pathologies, 60% were gill-related. Of these, about 25% appeared to have fungal diseases and another 15% had regions of gill lamellae that were eroded and necrotic. Other pathologies observed were: hemorrhagic areas on the

TABLE 3. BaP CONCENTRATION IN AQUARIA OF CLOSED-CIRCULATING SYSTEM[1]

Week of Exposure	Time of Sampling			
	1 hr	24 hr	96 hr	144 hr
1	21.9[2]	10.0	1.0	1.0
2	18.8	15.9	1.8	1.4
3	24.6	7.0	1.7	0.9
Mean ± S.E.	21.8 ± 4.5	11.0 ± 4.5	1.5 ± 0.44	1.1 ± 0.26

[1]System contaminated at the beginning of each week with 50 µg BaP per liter of water.
[2]All values in µg/l (ppb).

TABLE 4. MCA CONCENTRATION IN AQUARIA OF CLOSED-CIRCULATING SYSTEM[1]

Week of Exposure	Time of Sampling			
	1 hr	24 hr	96 hr	144 hr
1	61.2[2]	42.8	3.6	3.5
2	65.5	42.4	7.4	6.0
3	71.4	55.6	7.4	5.8
4	62.2	41.9	6.9	5.3
5	66.4	52.0	5.0	5.1
Mean ± S.E.	65.3 ± 4.0	47.7 ± 5.9	6.1 ± 1.7	5.1 ± 1.0

[1]System contaminated at the beginning of each week with 50 µg MCA per liter of water.
[2]All values in µg/l (ppb).

body surface 12%, hemorrhagic gills 8%, and lordosis and/or scoliosis 8%.

Pathological conditions observed in channel catfish that became ill or moribund while in the laboratory were similar to those observed in the sheepshead minnows. For example, about 77% of the abnormalities in catfish were gill-related. In contrast to the sheepshead minnows, 47% of the catfish cases that we classified under gill abnormalities were severe gill infestations of the monogenetic trematode Cleidodiscus. An apparent explanation for this occurrence is that 33% of these infestations of Cleidodiscus occurred on fish that were stressed because of ingesting high levels of PAH in their food regime. About 20% of the catfish sacrificed had hemorrhagic and/or aneuritic lesions on the gills.

These data concerning the types and incidence of lesions of sheepshead minnows and channel catfish maintained in the laboratory are important since they serve as baseline information required to accurately assess the effects of contaminates introduced into the systems. The question regarding whether the lesions observed may result from maintaining the fish in the laboratory is problematic since extensive data on the survival rates, types, and incidence of lesions in the fish in their normal environments are not available.

In an experiment in which 10 catfish were maintained in water contaminated on a weekly basis with 1.0 µg/l of BaP the fish remained healthy for approximately 7 months. During the next three month period, however, 4 of the fish presented with a similar pathology. In each case they became very scoliotic and/or lordotic and exhibited nervous disorders in their manner of movement. Most of the affected individuals also displayed abnormal melanocytic control and were much darker in color than normal fish. Radiographs (Fig. 4) illustrate the vertebral disorientations that occurred. Lesions of this type can result from inadequate nutrition (30) and we have on occasion observed scoliosis in catfish maintained as controls; however, the high incidence of these phenomena in the exposed fish that continued to feed normally, and had normal growth rates, suggests that a cause-effect relationship may exist between the lesions and the BaP exposure. These lesions, though not identical, are quite similar to those that appear to occur in teleosts as a result of exposure to Kepone (29).

Feeding Experiments

The tendency of PAH to adsorb to particulate matter makes it likely that scavenger feeding fish such as the channel catfish could be exposed to large amounts of PAH by ingestion. In an effort to reproduce this type exposure, channel catfish were fed diets containing known concentrations of BaP and MCA (Table 5).

In an experiment in which the fish were fed 1 mg BaP/gm food, 56% of the fish were dead in 24 hr and all but one had died by the 4th day of the experiment. Most of the fish exhibited a "spiraling" behavior just before becoming moribund. In a second experiment in which catfish were fed 1 mg BaP/gm food, all the fish lived until the 6th day and 60% had died by the 7th day. The fish continued to die until the remaining 5 were sacrificed on the 16th day.

TABLE 5. FEEDING EXPERIMENTS

EXPT. NO.	FOOD REGIME[1]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	BaP-H		-14	-1	-9					-1															
2	BaP-H						-2	-13			-1			-2		-2	*								
3	MCA-H								-1	-1	-1	-1	-1		-1	-1	-1		-6		-5	-2	*		
4	BaP-L													-8	-14	†									
5	MCA-L										-3	-2	-3	-4	-1										§

[1]Twenty-five fingerling channel catfish were used in each of these experiments. The food regime in experiments 1 and 2 (BaP-H) contained 1 mg BaP/g of food. In experiment 3 (MCA-H) the food was contaminated with 1 mg MCA/g of food. The contamination in experiment 4 (BaP-L) was 0.1 mg BaP/g food, and 0.1 mg MCA/g in experiment 5 (MCA-L).

*Experiment terminated and surviving fish were sacrificed.

†Surviving fish were fed uncontaminated food for 10 additional days and then sacrificed.

§2 additional fish died on day 27 and 1 on day 35. The experiment was terminated on the 36th day and the surviving fish were sacrificed.

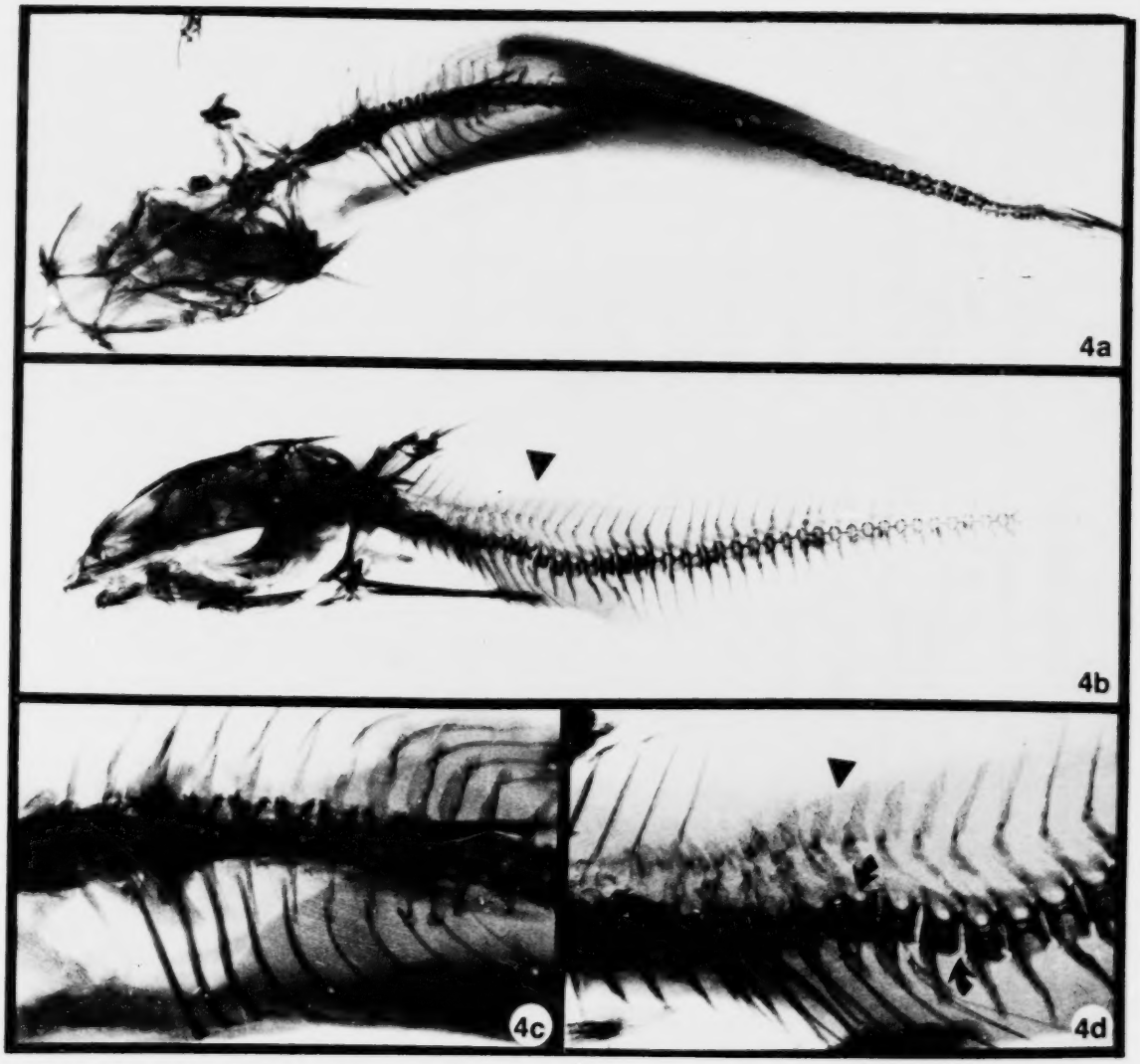

Figure 4. Vertebral disorientations in BaP exposed catfish. Images are positive prints of radiographs.
4a) Dorsal view. The vertebral column appears essentially normal. Mag. 2X.
4b) Lateral view. The disorientation of vertebra in the anterior half of the vertebral column is evident. Note the twisting of some of the vertebrae to an extent that the neural spines are out of the focal plane (arrowhead). Mag. 2X.
4c) Dorsal view. A higher magnification of the anterior region of the vertebral column. Mag. 5X.
4d) Lateral view. The disarticulation of many of the vertebrae is evident. Note that some vertebrae extend above adjacent vertebra and others have abnormally large spacing (arrows). The rotation of some of the vertebrae removes their neural spines from the plane of the image (arrowhead). Mag. 5X.

TABLE 6. BIOACCUMULATION OF ^{3}H-BENZO(A)PYRENE IN SHEEPSHEAD MINNOWS[1]

	Gills		Liver		GI Tract		Muscle	
Time Exposed	ng/gm[2] tissue	BF[3]	ng/gm tissue	BF	ng/gm tissue	BF	ng/gm tissue	BF
6 hr	0.06-0.42	2.3	0.15-0.59	4.9	0.58-0.73	8.2	0.01-0.06	0.5
12 hr	0.08-0.21	1.8	0.2 -1.6	1.0	0.89-1.9	18	0.02-0.05	0.6
24 hr	0.12-0.27	2.5	0.29-0.44	4.4	0.19-0.75	5.9	0.03-0.12	0.6
48 hr	0.20-0.26	2.9	0.34-0.60	6.4	1.41-1.60	19	0.04-0.08	0.7
96 hr	0.04-0.13	1.1	0.04-0.60	5.0	0.53-0.75	8	0.02-0.11	0.6

[1]Theoretical concentration of ^{3}H-BaP, 0.08 ng/ml water.
[2]Range of values determined from 3 experiments.
[3]BF = Bioaccumulation Factor: mean concentration of BaP in tissue divided by theoretical concentration of BaP in water.

TABLE 7. BIOACCUMULATION OF ^{3}H-METHYLCHOLANTHRENE IN SHEEPSHEAD MINNOWS[1]

	Gills		Liver		GI Tract		Muscle	
Time Exposed	ng/gm^{2} tissue	BF[3]	ng/gm tissue	BF	ng/gm tissue	BF	ng/gm tissue	BF
24 hr	1.6 -5.6	97	0.4 -8.2	132	6.4 -11.3	227	0.11-0.30	6
48 hr	0.2 -9.6	97	0.19-2.5	35	0.26-3.8	54	0.11-0.20	4
72 hr	1.0 -4.0	57	0.10-6.0	65	0.48-1.46	25	0.08-0.18	3
96 hr	0.65-3.0	49	2.9 -7.5	140	0.25-1.02	16	0.03-0.26	4
120 hr	3.3 -6.9	124	1.4 -8.5	135	0.12-0.68	10	0.01-0.04	1
144 hr	3.6 -4.0	86	1.3 -7.5	135	0.16-1.03	12	0.03-0.43	4
168 hr	0.56-12.7	189	0.46-0.5	13	0.09-0.26	5	0.02-0.04	1

[1]Theoretical concentration of ^{3}H-MCA, 0.037 ng/ml water.
[2]Range of values determined from 3 experiments.
[3]BF = Bioaccumulation Factor: mean concentration of MCA in tissue divided by theoretical concentration of MCA in water.

TABLE 8. BIOACCUMULATION OF ^{3}H-BENZO(A)PYRENE IN CHANNEL CATFISH[1]

Time Exposed	Gills ng/gm[2] tissue	Gills BF[3]	Liver ng/gm tissue	Liver BF	GI Tract ng/gm tissue	GI Tract BF	Muscle ng/gm tissue	Muscle BF
6 hr	0.12-0.62	4.7	0.60-3.0	21	0.13-1.3	7	0.14-0.30	2
12 hr	0.25-0.71	5.5	1.7 -2.7	4.4	0.53-2.3	14	0.23-0.26	3
24 hr	0.17-0.53	4.1	0.5 -2.8	25	0.09-1.6	11	0.26-0.43	4
48 hr	0.04-0.19	1.4	0.5 -3.5	26	0.3 -2.6	11	0.14-0.42	4
72 hr	0.07-0.16	1.5	0.17-2.0	14	0.25-1.3	10	0.19-0.66	5
96 hr	0.08-0.20	1.6	0.04-3.2	18	0.08-2.6	17	0.06-0.51	2
120 hr	0.04-0.46	3.4	0.04-0.12	1	0.03-0.85	6	0.11	1

[1]Theoretical concentration of ^{3}H-BaP, 0.08 ng/ml water.
[2]Range of values determined from either 3 or 5 experiments.
[3]BF = Bioaccumulation Factor: mean concentration of BaP in tissue divided by theoretical concentration of BaP in water.

TABLE 9. BIOACCUMULATION OF ^{3}H-METHYLCHOLANTHRENE IN CHANNEL CATFISH[1]

	Gills		Liver		GI Tract		Muscle	
Time Exposed	ng/gm^{2} tissue	BF[3]	ng/gm tissue	BF	ng/gm tissue	BF	ng/gm tissue	BF
24 hr	0.03-1.8	25	0.02-0.59	9	0.01-0.91	9	0.10-0.13	3
48 hr	0.07-0.16	3	0.01-0.20	14	0.04-1.32	14	0.05-0.10	2
72 hr	0.014-0.02	0.5	0.03-0.08	2	0.02-0.54	8	0.06-0.10	2
96 hr	0.01-0.06	1	0.05-1.20	14	0.01-0.10	2	0.06-0.2	3.5
120 hr	0.02	0.5	0.04-0.7	8	0.01-0.06	1	0.03-0.1	2
144 hr	0.07	2	0.20-0.40	8	0.02-0.04	1		
168 hr	0.06	1.5	0.14-0.6	10	0.02-0.08	1		

[1]Theoretical concentration of ^{3}H-MCA, 0.037 ng/ml of water.
[2]Range of values determined from 3 experiments.
[3]BF = Bioaccumulation Factor: mean concentration of MCA in tissue divided by theoretical concentration of MCA in water.

These fish were sluggish, not feeding, and their gills were infested with the monogenetic trematode Cleidodiscus. In a similar experiment in which catfish were fed 1 mg MCA/gm food, a slow mortality began to occur on the 8th day and continued until the 18th day when 6 of the remaining fish died. On the 20th day an additional 5 of the survivors died. In this experiment also, the fish became sluggish and heavily infested with trematodes simultaneously with the high mortality. When catfish were fed 0.1 mg BaP/gm food, they eventually became sluggish and fed poorly, but no mortality occurred until the 13th day when within 2 days 88% of the fish died. Somewhat similarly, when catfish were fed 0.1 mg MCA/gm food, the fish began to show the same signs after about a week. The first mortalities occurred on the 10th day, and by the 14th day 52% of the fish had died. In a control experiment in which catfish were maintained on a regime to which acetone had been added as in the PAH contaminated food, the fish continued to feed normally during a two-month period. They were thereafter maintained on a regular diet for an additional seven months with no visible effects.

These results seem to indicate that BaP fed at the level of 1 mg/gm food is toxic to the channel catfish and that MCA fed at the same level is toxic to a somewhat lesser degree. Further, either of these compounds fed at the lower level (0.1 mg/gm food) seem to be less toxic but still stressful enough to allow parasitic infestation and eventual death.

These results, although preliminary, suggest a need for more work to clarify the toxic effects of PAH on channel catfish. Of particular interest is the indication that ingestion of nontoxic amounts of PAH may cause enough stress to lower resistance sufficiently to allow secondary infestations by viruses, bacteria, or parasites.

Bioaccumulation Studies

The range of variability in the quantities of PAH at each time period makes interpretation of these data problematic (Tables 6-9). The technique employed in the studies allows for some amount of experimental error (e.g., wet weights of tissues in milligram quantities must be determined); however, previous experience with these techniques suggests that this could not account for all the variability observed. Consequently, some of the experiments were carefully repeated to provide as many as six data points at one exposure time. The variability persisted, suggesting that rather large and capricious variabilities in bioaccumulation actually occur.

When the bioaccumulation of BaP and MCA are considered in both sheepshead minnows and catfish, the values for the liver and GI tract are consistently the highest. The result in the liver could be anticipated because of the major role this organ plays in the metabolism, detoxification, and storage of exogenous compounds. The relatively high levels of PAH in GI tract perhaps would not have been predicted. The tendency for PAH to adhere to surfaces rather than remain in solution in the water column may be significant to this result. For example, this may mean that the major route of exposure to the labelled compound was via the GI tract as a result of the

fish ingesting debris from the aquaria with adherent labelled PAH.

The level of label in the gills was intermediate, lower than the liver and GI tract but consistently higher than the levels observed in skeletal muscle. This likely results from the fact that the gills are highly vascularized and have an excretory role.

In each case the level of PAH in the skeletal muscle was significantly lower and less variable than that observed in the other tissues. Since muscle tissue is not directly involved in the metabolism of exogenous compounds such as PAH, a lower level of label could be expected.

No consistent trend toward either an increase or decrease in the level of the label was observed during the time the fish were exposed to the labeled compound. This remained true even in some experiments in which fish were exposed for as long as 12 days.

The basic pattern and the absolute values of label in the tissue were essentially similar for both species of fish with respect to BaP. In the catfish, the MCA experiments were also comparable to the BaP results; however, in the MCA experiments with sheepshead minnows, the levels were significantly higher. It is possibly important in this respect that MCA seems to remain in the water column of the closed-circulating systems at higher levels than BaP (Tables 3-4). It is our casual observation that MCA is also more soluble in the acetone carrier used to contaminate the systems. This observation does not, however, explain why a similar increase in label was not observed in the catfish experiments with MCA.

When the bioaccumulation experiments are considered in toto, one reasonable explanation for the results is that the major route of contamination is by ingestion rather than uptake through contact with the labeled compound suspended in the water column. In this method of exposure, one would predict that the level of radioactivity in the tissues of the fish would be related more to whether a particular fish had recently ingested significant amounts of the contaminant than how long it had been exposed to the contaminated water. Experiments in which fish will be fed food containing labeled contaminate are planned in an attempt to clarify this point. Also, autoradiographic studies of tissues from these experiments are currently being conducted to elucidate the route of exposure.

Due to the techniques used in these experiments, the results indicate only the tissue location of the radioactive label and provide no indication of the biochemical changes that may have occurred in the parent compound due to metabolic processes. Valuable information of this type must await more sophisticated biochemical studies.

Tissue Culture Studies

The basic mechanisms of chemical carcinogenesis function at the cellular level and remain incompletely understood. With the long term goal of studying these phenomena in fish tissue culture cells, we have karyotyped an

established fin fibroblastic cell line designated SHF (19). Since many teleosts possess large groups of morphologically similar chromosomes, it was necessary to modify existing techniques for C-banding mammalian cells so that C-bands could be observed on SHF cells before they could be adequately karyotyped for use in an experimental system. A publication concerning the development of this technique and its successful application to three different species of fishes is currently in press (31).

A complete karyotyping of SHF cells has been completed and the results have been submitted for publication (32). It appears from this study that the SHF culture has a relatively stable karyotype with a modal chromosome number of 48. Essentially no morphological changes were observed in the SHF cell chromosomes, so this cell line seems to provide an excellent model for in vitro studies for carcinogenicity and mutagenicity. Currently, preliminary experiments are in progress to determine the toxicity level of BaP with respect to these cells so chronic exposures can be accomplished.

Histological Studies

To date, histological examinations have been conducted on 250 (3%) of the approximately 8,000 specimen collected. About 70% of these specimen were sheepshead minnows, and the tissues examined most extensively were gills, liver, GI tract, and skeletal muscle. To a lesser extent gonads, kidneys, and heart tissues were also examined. Some of the fish represent random sampling of the feral population, and others were taken from either control or contaminated exposure systems. However, a majority were from moribund fish or fish that appeared to be ill. Most of the histopathic lesions observed were in the gills. These included hyperplasia, aneurisms, necrosis, and parasitic infestations. These data are not sufficient to determine whether the incidence of any of the lesions could be consistently associated with PAH exposure. This determination will have to await collection of additional data concerning the incidence of the various lesions in feral laboratory maintained controls, and experimentally exposed sheepshead minnows and channel catfish.

A library of slides representing the major organs and tissues from both sheepshead minnows and channel catfish has been prepared to serve as a baseline of "normal" tissues for reference when studying tissues from the species that were maintained under long-term exposures. We have also cataloged representative slides illustrating the lesions observed in sick and moribund fish sacrificed from both control and contaminated exposure systems.

Because of the continued need for more sensitive indices of the effects of pollutants on the aquatic environment, we conducted the following morphological studies in addition to routine histology. The morphology of the gills from both healthy and sick or moribund fish are being studied with the scanning electron microscope, and white blood cells of healthy and diseased sheepshead minnows are being studied by light and transmission electron microscopy. The obvious need to understand the effects of pollutants on fish during the preadult stages of their life cycle has prompted a careful study of the embryologic development of the sheepshead minnow. The results

of these studies will be published subsequent to submission of this report.

Spontaneous Tumors

Two lesions with a tumor-like appearance were observed in the sheepshead minnow feral population. In the first case, when the specimen was necropsied, a large mass was observed protruding from the liver. Its color and texture was obviously different from a normal liver; however, attempts at histological analysis disclosed that the tissue had experienced such severe autolytic damage that its histological nature was completely obscured.

In a second case, a tumor-like mass was observed in a sheepshead minnow shortly after it was brought into the laboratory. The lesion was a relatively large mass of soft tissue protruding from the body surface in the isthmus region between the pectoral fins.

At low magnification, the tissue has the appearance of a tumor in that it consists of a randomly oriented connective tissue stroma that forms sinus-like cavities containing a population of loosely oriented and apparently pleomorphic cells (Fig. 5a). However, at higher magnification, it becomes clear that the loosely associated cells are myxosporodian spores that appear pleomorphic because of their various orientations (Fig. 5b). At an even higher magnification, the morphology of the spore and its two internal polar capsules may be observed (Fig. 5c). The histozoic and possibly cytozoic nature of this parasite is illustrated by the close association of spores with striated muscle fibers that extended in an irregular orientation through some regions of the cyst (Fig. 5d). Although not prevalent in the fishes of the Gulf, this type lesion has been observed in the sheepshead minnow and is likely the myxosporidian Myxobolus lintoni (33). It has been suggested that the incidence of this parasite may be increased by pollution or other forms of stress (33). The lesion is illustrated and described in some detail in this report in order to point out that these type lesions should not be confused with true neoplasms.

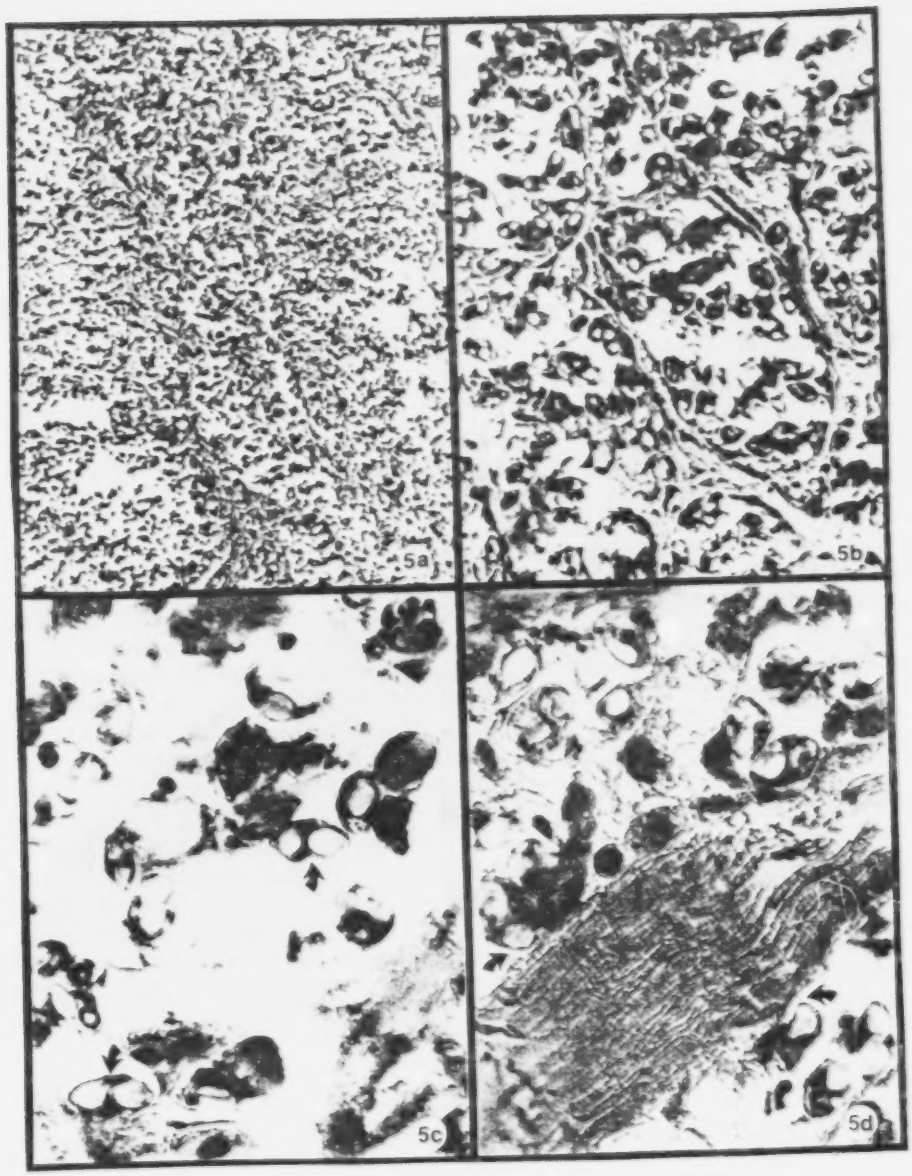

Figure 5. Myxosporidian cyst in the sheepshead minnow. H & E stained sections viewed with bright field optics.
5a) At low magnification the cyst has a tumor-like appearance. There is a connective stroma with a parenchyma of loosely associated cells that appear to be pleomorphic. Mag. 160X.
5b) At somewhat higher magnification the vacuolated nature of the structures within the stroma is evident (arrows). Mag. 634X.
5c) At even higher magnification it is evident that these structures are flattened ovoidal myxosporidian spores. In properly oriented spores the two anteriorly located polar capsules are evident (arrows). Mag. 4800X.
5d) Scattered sparsely throughout this lesion are randomly oriented muscle fibers. Mag. 1585X.

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